

## Anisaldehyde and Veratraldehyde Acting as Redox Cycling Agents for $\text{H}_2\text{O}_2$ Production by *Pleurotus eryngii*

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The existence of a redox cycle leading to the production of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in the white rot fungus *Pleurotus eryngii* has been confirmed by incubations of 10-day-old mycelium with veratryl (3,4-dimethoxybenzyl) and anisyl (4-methoxybenzyl) compounds (alcohols, aldehydes, and acids). Veratraldehyde and anisaldehyde were reduced by aryl-alcohol dehydrogenase to their corresponding alcohols, which were oxidized by aryl-alcohol oxidase, producing  $\text{H}_2\text{O}_2$ . Veratric and anisic acids were incorporated into the cycle after their reduction, which was catalyzed by aryl-aldehyde dehydrogenase. With the use of different initial concentrations of either veratryl alcohol, veratraldehyde, or veratric acid (0.5 to 4.0 mM), around 94% of veratraldehyde and 3% of veratryl alcohol (compared with initial concentrations) and trace amounts of veratric acid were found when equilibrium between reductive and oxidative activities had been reached, regardless of the initial compound used. At concentrations higher than 1 mM, veratric acid was not transformed, and at 1.0 mM, it produced a negative effect on the activities of aryl-alcohol oxidase and both dehydrogenases.  $\text{H}_2\text{O}_2$  levels were proportional to the initial concentrations of veratryl compounds (around 0.5%), and an equilibrium between aryl-alcohol oxidase and an unknown  $\text{H}_2\text{O}_2$ -reducing system kept these levels steady. On the other hand, the concomitant production of the three above-mentioned enzymes during the active growth phase of the fungus was demonstrated. Finally, the possibility that anisaldehyde is the metabolite produced by *P. eryngii* for the maintenance of this redox cycle is discussed.

White rot fungi are the best lignin degraders among all known organisms (29). A common characteristic of these fungi is their ability to produce large amounts of nitrogen-free secondary metabolites from the shikimic acid and cinnamic acid pathways (41, 42). Although the biological significance of this fact remains uncertain, the production of different compounds could make possible a variety of functions which could be related to lignocellulose degradation.

Among these secondary metabolites, veratryl (3,4-dimethoxybenzyl) alcohol has been the subject of many studies, as it is a substrate of lignin peroxidase (LiP), which is a major enzyme involved in lignin degradation. In addition to LiP, manganese-dependent peroxidase and laccase are enzymes catalyzing lignin breakdown (37). Synthesis of veratryl alcohol from glucose in several ligninolytic fungi, such as *Phanerochaete chrysosporium* (40), *Pycnoporus cinnabarinus* (22), *Trametes versicolor* (26), *Phlebia radiata* (24), and *Bjerkandera adusta* (5), has been described. Although the main function of veratryl alcohol remains uncertain, several pieces of evidence indicate that it has a definite role in ligninolysis. It stimulates the production of LiP both by *P. chrysosporium* (8, 32) and by *P. radiata* (36), this role being in dispute in the case of *P. chrysosporium* (2); it prevents the inactivation of LiP by excess  $\text{H}_2\text{O}_2$  and closes the catalytic cycle of the enzyme (19, 43); and the veratryl cation radical produced by the action of LiP can act as an electron carrier for the oxidation of lignin (11, 21). Much less is known about other aromatic compounds produced by white rot fungi; for example, the production of anisaldehyde in white rot fungi such as *Ischnoderma benzoinum* (1), *Dichomitus squaleus* (12), *Armillaria mellea* (12), *B. adusta*

(5), and several species of the genus *Pleurotus* (18) has been reported, but its function has not been demonstrated.

The generation of  $\text{H}_2\text{O}_2$  is another common characteristic of white rot fungi (23, 31).  $\text{H}_2\text{O}_2$  is necessary for the activity of ligninolytic peroxidases; however, white rot fungi produce  $\text{H}_2\text{O}_2$  under culture conditions in which these enzymes have not been detected (15). Therefore, there must be some other function(s) for  $\text{H}_2\text{O}_2$  in white rot fungi. It has been shown that cellulose can be degraded by the nonenzymatic mechanism of Fenton's reagent, in which the interaction of  $\text{Fe}^{2+}$  and  $\text{H}_2\text{O}_2$  produces hydroxyl radicals ( $\text{OH}\cdot$ ) (20). Moreover, it has been reported that lignin degradation is markedly suppressed by agents that scavenge  $\text{OH}\cdot$  and that this radical reacts with both monomeric (13) and dimeric (30) models of lignin. In addition, low-molecular-weight molecules are able to penetrate into the lignocellulosic matrix of wood cell walls, which are inaccessible to enzymes, thus initiating the degradative process (6, 7).

A connection between some secondary metabolites derived from the cinnamate pathway and the production of  $\text{H}_2\text{O}_2$  can be established through two extracellular oxidases reported to exist in white rot fungi: glyoxal oxidase (27) and aryl-alcohol oxidase (AAO) (10). Glycolaldehyde and aromatic alcohols are oxidized by glyoxal oxidase and AAO, respectively, producing  $\text{H}_2\text{O}_2$ . AAO also generates  $\text{H}_2\text{O}_2$  in oxidizing aromatic aldehydes, but to a much lesser extent (15). While further metabolism of glyoxal oxidase oxidation products has not yet been shown to occur in white rot fungi, one of the main reactions occurring with the aromatic aldehydes and acids produced by AAO (or derived from lignin degradation) is the intracellular reduction to their corresponding alcohols (9, 33, 39). This fact led us to the possible implication of dehydrogenases, which catalyze the reduction of aromatic acids and aldehydes, in the process of  $\text{H}_2\text{O}_2$  production by recycling oxidized AAO substrates. We recently reported the first evi-

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dence about the existence of a cyclic system for  $\text{H}_2\text{O}_2$  production in *Pleurotus eryngii*, involving successive oxidation and reduction reactions of either benzyl alcohol, benzaldehyde, or benzoic acid (16, 17). In that system, AAO produced  $\text{H}_2\text{O}_2$  by oxidizing benzyl alcohol to benzaldehyde and, to a lesser extent, benzaldehyde to benzoic acid. Reduction of benzaldehyde and benzoic acid was carried out by aryl-alcohol dehydrogenase (AAD) and aryl-aldehyde dehydrogenase (AADD), respectively.

On the basis of the wide substrate specificity of AAO, the aim of the present study is to check the operational validity of that  $\text{H}_2\text{O}_2$ -producing redox system with other aromatic alcohols, aldehydes, and acids. For this purpose, veratryl and anisyl compounds were chosen.

## MATERIALS AND METHODS

**Organism and culture conditions.** *P. eryngii* IJFM A169 (Fungal Culture Collection of the Centro de Investigaciones Biológicas, Madrid, Spain) was maintained at 4°C on 2% malt extract agar. Inocula were prepared by cultivating the fungus under the conditions used for growth and then homogenizing 10-day-old mycelium. The dry weight of inoculum was 0.1 g per 100 ml of medium. Mycelial pellets were produced at 28°C in shaken (150 rpm) 250-ml conical flasks with 100 ml of modified Czapek Dox medium containing 10 g of glucose per liter, 2 g of ammonium tartrate per liter, and 1 g of yeast extract per liter (15).

**Incubation with aromatic compounds.** After 9 to 10 days of growth, the culture liquid was eliminated by filtration and mycelia were washed three times with distilled water and resuspended in 100-ml aqueous solutions of aromatic compounds at different concentrations. Incubations were performed in 250-ml conical flasks, at 28°C for up to 24 h. Control experiments were carried out in the absence of aromatic compounds.

Samples were taken from two replicate flasks, filtered, and pooled. For analysis of  $\text{H}_2\text{O}_2$  and aromatic compounds, samples were heated at 80°C for 30 min, to avoid changes in their concentrations due to enzyme activities, and  $\text{H}_2\text{O}_2$  assays were done immediately after they were cooled. For enzyme assays, samples were dialyzed overnight against 20 mM sodium phosphate buffer, pH 6.0.

**Enzyme assays.** Direct estimations of enzyme activities were done with the extracellular liquid. AAO (EC 1.1.3.7) activity was measured spectrophotometrically as the oxidation of veratryl alcohol to veratraldehyde ( $\epsilon_{310}$  of  $9,300 \text{ M}^{-1} \text{ cm}^{-1}$ ) (15). The reaction mixture contained 0.1 M sodium phosphate buffer, pH 6.0, and 5 mM veratryl alcohol. AAD (EC 1.1.1.91) and AADD (EC 1.2.1.29) activities were measured by using veratraldehyde and veratric acid as the substrate, respectively. The reaction mixtures contained 0.2 mM substrate and 0.2 mM NADPH in 50 mM bistris-propane-HCl buffer, pH 6.0 (34). The decrease in  $A_{365}$ , indicating NADPH oxidation ( $\epsilon_{365}$  of  $3,510 \text{ M}^{-1} \text{ cm}^{-1}$ ), was monitored against blanks without substrates. Absorbance at 365 nm rather than at 340 nm (the maximum for NADPH) was used to avoid interferences with the absorbance maximum of veratraldehyde (310 nm).

Indirect estimations of AAO, AAD, and AADD activities were carried out by incubating the washed mycelium for 1 h with 100-ml aqueous solutions of 4 mM veratryl alcohol, 4 mM benzaldehyde, and 0.5 mM veratric acid, respectively. Incubation conditions and sample treatment were as described above. Veratryl alcohol consumption and production of both benzyl alcohol and veratraldehyde were measured by high-performance liquid chromatography (HPLC) as described below.

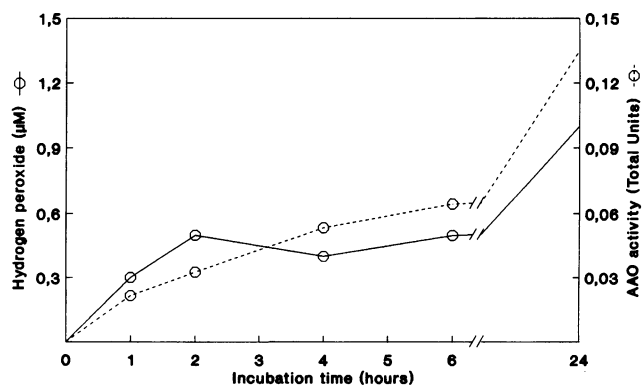


FIG. 1. Time courses of  $\text{H}_2\text{O}_2$  and AAO production by 10-day-old *P. eryngii* pellets in the absence of aromatic compounds. Total AAO activity is that detected in 100-ml incubation solutions.

International units (micromoles per minute) of enzyme activity were used.

**Analytical techniques.** The glucose content in the culture liquid was assayed as reducing sugars by the Somogyi-Nelson method (35).

$\text{H}_2\text{O}_2$  concentration was determined by using peroxidase and phenol red (38). The reaction mixture contained 0.01% (wt/vol) phenol red, 2.5 U of horseradish peroxidase (type II; Sigma) per ml, and 0.1 M sodium phosphate buffer, pH 6.0. After the addition of NaOH (0.2 M final concentration), the  $A_{610}$  was read. Samples preincubated with 30 U of catalase (Sigma) per ml were used as blanks. A standard curve of  $\text{H}_2\text{O}_2$  was prepared with dilutions of Perhydrol (Merck) processed in the same way. The  $\text{H}_2\text{O}_2$  concentration in the commercial solution was calculated by using an  $\epsilon_{230}$  of  $81 \text{ M}^{-1} \text{ cm}^{-1}$ .

Quantitative determinations of aromatic compounds were performed by HPLC with standard calibration curves for each compound. Samples (20 µl) were injected into a Merck-Hitachi HPLC system equipped with a Spherisorb S50DS2 column (Hichrom). These analyses were carried out at 30°C with a flow rate of  $1 \text{ ml min}^{-1}$  and with methanol-10 mM phosphoric acid (30:70) as the eluent. The UV detector was operated at 254 nm.

The experiments whose results are shown in the figures were all repeated two or more times to confirm that the results were reproducible ( $\pm 16\%$ ). Representative data are presented.

## RESULTS

In keeping with our previous reports (16, 17), incubations of *P. eryngii* with aromatic compounds were carried out in aqueous solutions and 10-day-old pellets grown in Czapek Dox medium were used. Figure 1 shows the extracellular AAO activity and  $\text{H}_2\text{O}_2$  production over a 24-h period in the absence of aromatic compounds (control experiment). AAD and AADD activities were not detected in the extracellular liquid.

Initial studies were carried out with the veratryl series (alcohol, aldehyde, and acid). The effects of different concentrations (0.5 to 4.0 mM) of either veratryl alcohol, veratraldehyde, or veratric acid on  $\text{H}_2\text{O}_2$  production are shown in Fig. 2. As can be seen, higher levels of  $\text{H}_2\text{O}_2$  than those obtained in the control experiment (Fig. 1) were found in all cases except with veratric acid. At 2.0 and 4.0 mM, the acid was not transformed at all (data not shown), whereas at 0.5 and 1.0 mM,  $\text{H}_2\text{O}_2$  was observed only at the end of the incubation period. Initially, levels of veratryl compounds changed rapidly

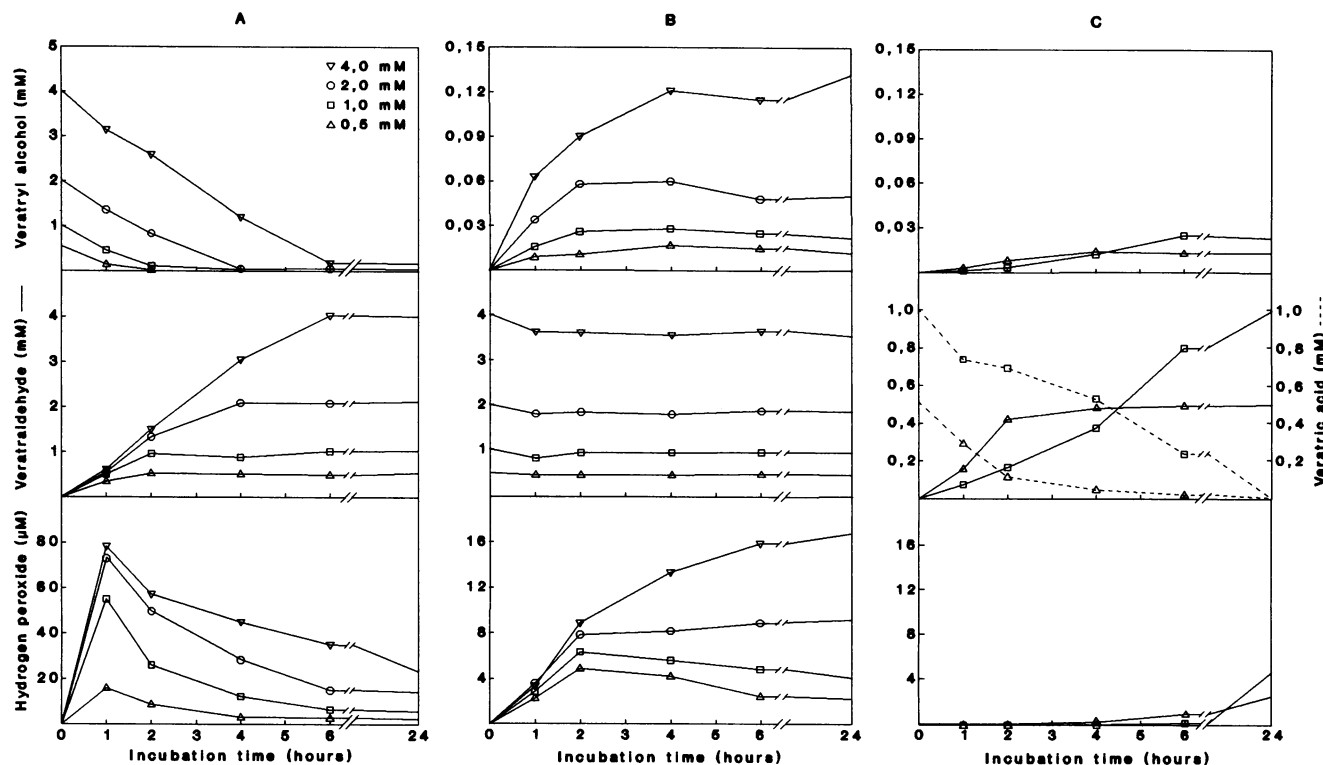


FIG. 2. Incubation of 10-day-old *P. eryngii* pellets in aqueous solutions of 0.5 to 4.0 mM veratryl alcohol (A), veratraldehyde (B), and veratric acid (C). H<sub>2</sub>O<sub>2</sub> production and veratryl compound metabolism are shown.

until steady values were attained, indicating that equilibrium between oxidative and reductive activities had been reached. In the alcohol and aldehyde solutions only trace amounts of veratric acid were found over the full study period, in agreement with the very low activity on veratraldehyde that has been reported for AAO in vitro (15). At equilibrium, around 94% of veratraldehyde and 3% of veratryl alcohol (referred to the initial molar concentration of the compound added) and trace amounts of veratric acid were found in all experiments. Similar percentages of the veratryl compounds were found in parallel incubations of the fungus with 0.2, 0.1, and 0.05 mM veratraldehyde. At the end of the incubation period, the H<sub>2</sub>O<sub>2</sub> molar concentration was always about 0.5% of the initial concentration of veratryl compounds. No significant differences between the amounts of extracellular AAO in the presence of veratryl compounds (data not shown) and in the control experiment (Fig. 1) were observed. However, an activity of 1.27 total units can be estimated from the veratraldehyde produced after 4 h from 4 mM veratryl alcohol (Fig. 2A) (the actual activity value being higher, since a portion of the aldehyde produced was reduced by AAD). This observation indicated that, during incubation of the washed mycelium, most of the AAO remained associated with the fungus.

The activity of AAO on veratryl alcohol is well illustrated in Fig. 2A. A ratio close to 1 between the veratryl alcohol consumed and the veratraldehyde produced was obtained for all samples, corresponding with the expected stoichiometry (1:1) of the reaction catalyzed by AAO (14). However, H<sub>2</sub>O<sub>2</sub> levels were always lower than those expected on the basis of the high alcohol oxidation rate observed. Furthermore, after the first hour H<sub>2</sub>O<sub>2</sub> levels began to gradually decrease until steady values were reached, even when AAO activity had not

yet declined. These results confirmed the existence of a system that was reducing H<sub>2</sub>O<sub>2</sub> (17). Therefore, an equilibrium between that system and AAO must be attained to keep H<sub>2</sub>O<sub>2</sub> at constant levels in the same way that dehydrogenases and AAO together regulate the concentration of veratryl compounds. On the other hand, Fig. 2B shows the reduction of veratraldehyde as a result of AAD activity. It is clear that, with the aldehyde being the main product at equilibrium, as the alcohol was produced by AAD it was rapidly oxidized by AAO, producing H<sub>2</sub>O<sub>2</sub>. Since some of this H<sub>2</sub>O<sub>2</sub> was at the same time being reduced (Fig. 2A), it can be assumed that H<sub>2</sub>O<sub>2</sub> time courses levelled off because of veratraldehyde redox cycling.

As mentioned above, veratric acid was not transformed at all when initial concentrations higher than 1 mM were used. This could be explained in terms of veratric acid toxicity, as has already been described for benzoic acid at a high concentration (10 mM), which impeded the consumption of the glucose present in the culture medium (17). This toxicity could also explain why in the experiments with veratric acid concentrations up to 1 mM, the production of veratraldehyde by AAD was negatively correlated to veratric acid concentration (Fig. 2C). Moreover, the fact that no H<sub>2</sub>O<sub>2</sub> was detected until the acid concentration was considerably decreased, even in the presence of veratryl alcohol, suggested that veratric acid could be inhibiting the extracellular AAO (it was determined that the activity of the peroxidase used in the H<sub>2</sub>O<sub>2</sub> assay was not affected by 1 mM veratric acid). If this was true, accumulation of veratryl alcohol should have been observed at the beginning of the experiment unless AAD activity was decreased (probably as a consequence of the acid toxicity). In order to verify that AAO and AAD activities were affected by veratric acid, *P. eryngii* was simultaneously incubated with veratric acid and

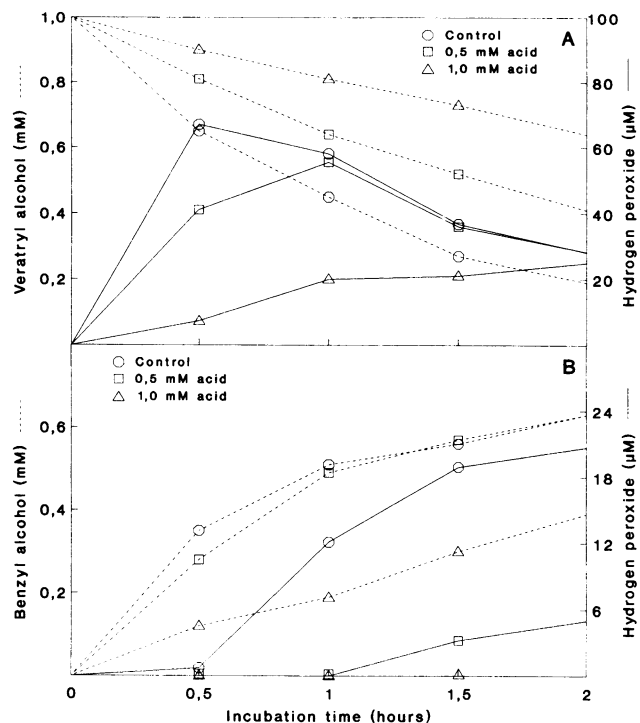


FIG. 3. Influence of 0.5 and 1.0 mM veratric acid on AAO and AAD activities. Ten-day-old *P. eryngii* pellets were incubated with 1.0 mM veratryl alcohol (A) and 4.0 mM benzaldehyde (B). AAO activity is shown as veratryl alcohol disappearance (A) and as H<sub>2</sub>O<sub>2</sub> production from both veratryl alcohol (A) and benzyl alcohol (B) oxidations. AAD activity is illustrated by the benzyl alcohol produced from benzaldehyde (B).

either benzaldehyde or veratryl alcohol. Since benzyl alcohol was the product accumulated at equilibrium when benzyl compounds were used (17), benzaldehyde was used to estimate AAD activity. Control experiments were performed in the absence of acid. Results concerning veratryl alcohol disappearance, H<sub>2</sub>O<sub>2</sub> generation, and benzyl alcohol production are shown in Fig. 3. Firstly, in experiments with veratryl alcohol (Fig. 3A), both parameters showing AAO activity dropped as the initial veratric acid concentration increased. Moreover, a greater AAO inhibition took place when the alcohol level was lower, as shown in experiments with benzaldehyde (Fig. 3B). For example, after 1 h of incubation, no H<sub>2</sub>O<sub>2</sub> was found with 0.5 mM veratric acid, even though an amount of benzyl alcohol similar to that in the control experiment had been produced. Secondly, the negative influence of veratric acid on AAD activity was also demonstrated, as shown by the comparison of the three concentrations of benzyl alcohol at 0.5 h in Fig. 3B.

If there is cooperation between AAO and dehydrogenases to produce H<sub>2</sub>O<sub>2</sub> on a constant basis, the concomitant production of these enzymes would be expected. To check this hypothesis, the time-dependent production of AAO, AAD, and AADD by *P. eryngii*, when cultivated in Czapek Dox medium, was investigated over a 26-day period. Under these conditions, the carbon source, which was the limiting growth factor, was depleted on day 13. Figure 4 reveals that the production of the three enzymes occurred during the active phase of growth. AAO was found to be both extracellular and cell bound, and both of these activities increased for the first 12 days. Then, the cell-bound activity gently decreased until the end of the

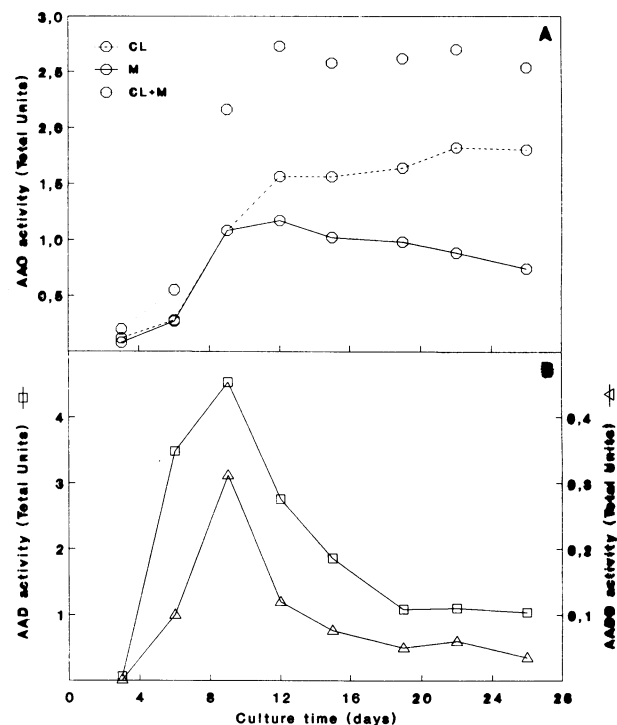


FIG. 4. Time courses of AAO (A) and AAD and AADD (B) activities over a 26-day period of *P. eryngii* cultivation in Czapek Dox medium. Activities were assayed directly in the culture liquid (CL; dashed line) and indirectly in the mycelium (M; continuous line) as described in Materials and Methods.

experiment, while at the same time the extracellular activity increased, with the total activity remaining constant (Fig. 4A). AAD and AADD activities were found only in the mycelium, reaching their maximal levels on day 9 (Fig. 4B). Since dehydrogenase activities depend on a source of reducing equivalents, the sharp decrease of activity levels then observed (measured indirectly by the mycelium reduction of benzaldehyde and veratric acid) could have arisen not only from a decrease of enzyme quantities but also from a decrease of NADPH levels, both probably due to the near exhaustion of glucose.

After H<sub>2</sub>O<sub>2</sub> production from veratryl compounds in *P. eryngii* was checked, further experiments were done with the anisyl series. These incubations were carried out with 9-day-old *P. eryngii* mycelium and 0.5 mM anisyl alcohol, anisaldehyde, and anisic acid. H<sub>2</sub>O<sub>2</sub> levels in the absence of anisyl compounds were similar to those shown in Fig. 1. As shown in Fig. 5, these compounds exhibited the same metabolic behavior as veratryl compounds, with the aldehyde being accumulated at equilibrium. The main difference was that at equilibrium neither alcohol nor acid was detected. This was probably due to the fact that AAO has much higher enzymatic efficiency ( $V_{\max}/K_m$ ) for anisyl alcohol (5,200) than for veratryl alcohol (300) and that its activity on anisaldehyde is negligible (15). On the other hand, time courses of H<sub>2</sub>O<sub>2</sub> production were similar to those found with veratryl compounds, showing again the existence of an uncharacterized H<sub>2</sub>O<sub>2</sub>-reducing system (Fig. 5A) and the negative effect of the acid on H<sub>2</sub>O<sub>2</sub> production (Fig. 5C).

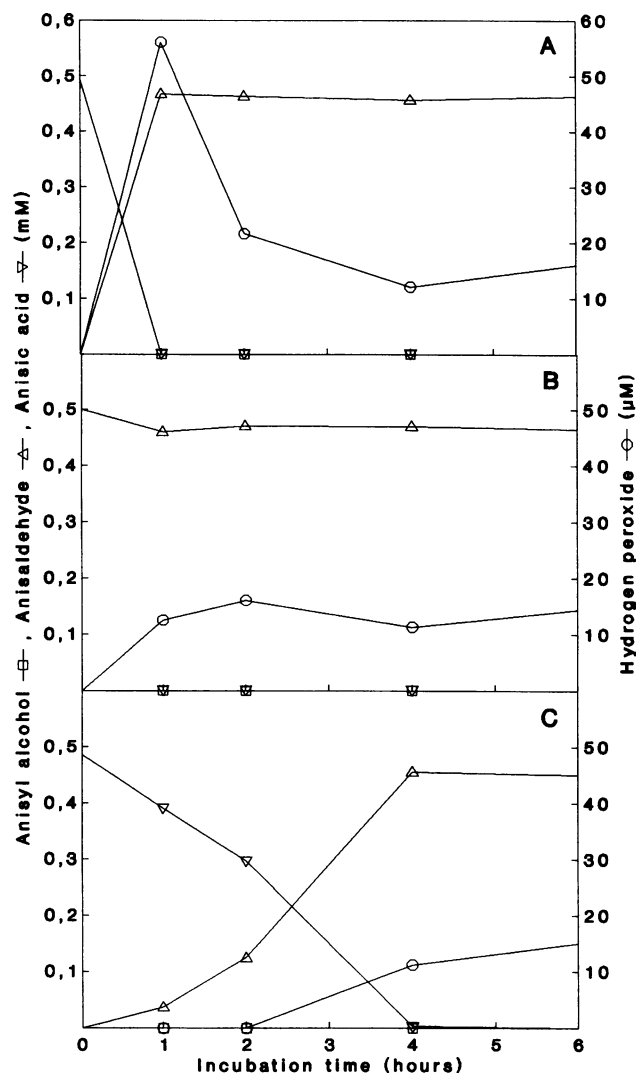


FIG. 5. Incubation of 9-day-old *P. eryngii* pellets in aqueous solutions of 0.5 mM anisyl alcohol (A), anisaldehyde (B), and anisic acid (C). H<sub>2</sub>O<sub>2</sub> production and anisyl compound metabolism are shown.

## DISCUSSION

The results presented in this study confirm the validity of the previously proposed redox system for H<sub>2</sub>O<sub>2</sub> production in *P. eryngii* (17). Such a system, which was first evidenced by the use of benzyl compounds, can now be extended both to more-representative secondary metabolites of *Pleurotus* species and to aromatic compounds more closely related in structure to lignin.

From the results shown in Fig. 2, it is inferred that H<sub>2</sub>O<sub>2</sub> is continuously produced by veratraldehyde redox cycling. This involves the initial two-electron reduction of the aldehyde by AAD and the subsequent electron transfer from veratryl alcohol to O<sub>2</sub> catalyzed by AAO. On the basis of the fact that AAD is an NADPH-dependent enzyme (18, 34), it is clear that extracellular O<sub>2</sub> reduction is achieved at the expense of cellular reducing equivalents. Therefore, H<sub>2</sub>O<sub>2</sub> production by redox cycling implies a continuous drain of NADPH, which does not seem to be a problem for white rot fungi since there is a carbon excess in their natural substrates. Of proven relevance is the

requirement of an alternative carbon source for lignin degradation (28).

We have termed veratraldehyde and anisaldehyde redox cycling agents by analogy with a large number of compounds which are supposed to exert toxic effects in biological systems by generating reduced oxygen species (25). In general, these compounds are reduced by intracellular NADPH-dependent enzymes, predominantly in one-electron transfer reactions, before they in turn reduce oxygen to the superoxide anion radical. The production of H<sub>2</sub>O<sub>2</sub> by *P. eryngii* from aromatic aldehydes involves, in addition, the action of the extracellular AAO, since aromatic alcohols by themselves are not able to reduce oxygen. While superoxide anion radical production by redox cycling is mainly an intracellular process, the requirement of the extracellular AAO for the production of H<sub>2</sub>O<sub>2</sub> by a similar mechanism avoids the presence of H<sub>2</sub>O<sub>2</sub> and derived radicals inside the cells and places the formation of H<sub>2</sub>O<sub>2</sub> in the site where it is required.

Our interest in demonstrating some increase of H<sub>2</sub>O<sub>2</sub> basal levels in the presence of anisyl compounds arose from two previously reported findings. Firstly, anisyl alcohol is the best AAO substrate among substituted benzyl alcohols (15). Secondly, anisaldehyde is the major extracellular aromatic metabolite produced by six different species of the genus *Pleurotus*, including *P. eryngii* (18). In the case of *Pleurotus pulmonarius*, which was more exhaustively studied, anisaldehyde levels are more than 1 order of magnitude higher than those of any other metabolite, regardless of the culture time. In the present study, the first evidence of H<sub>2</sub>O<sub>2</sub> production by anisaldehyde redox cycling is provided (Fig. 4). Taking these observations together, it is quite likely that the physiological role of the anisaldehyde produced by *Pleurotus* species is the maintenance of a redox cycle performed by AAD and AAO in order to produce H<sub>2</sub>O<sub>2</sub>. The same function has been recently suggested for the chlorinated aryl alcohols produced by a *Bjerkandera* strain (4).

It has been previously reported that the alcohol was the predominant product at equilibrium when *P. eryngii* was incubated with 5 mM benzaldehyde and benzyl alcohol (17). With the use of a much lower concentration (0.2 mM), a relatively high AAO activity on benzaldehyde, producing benzoic acid, was demonstrated. Although AAO activity on benzaldehyde was shown to be 86 times higher than that on veratraldehyde (15), the possibility of finding larger amounts of veratric acid has been investigated in the present study by reducing the initial veratraldehyde concentration to 0.2, 0.1, and 0.05 mM. As mentioned above, percentages of veratryl compounds similar to those observed with higher initial concentrations were found at equilibrium, with trace amounts of veratric acid being sporadically detected. Therefore, in spite of the existence in *P. eryngii* of AADD catalyzing aromatic acid reduction, the lack of a specific aryl-aldehyde oxidase relegates H<sub>2</sub>O<sub>2</sub> production by aromatic acid redox cycling to those acids, such as benzoic acid, whose corresponding aldehydes can be oxidized by AAO (15). Benzoic acid has been detected in the extracellular medium of the six *Pleurotus* species studied by Gutiérrez et al. (18). Veratric acid has been reported in wood degraded by white rot fungi (3). Reduction of aromatic acids to their corresponding alcohols is a well-known metabolic pathway carried out by white rot fungi. Schoemaker et al. (39) suggested that the function of these reductive reactions could be the production of more easily metabolizable compounds. We are showing now that the reduction of aromatic acids leads to H<sub>2</sub>O<sub>2</sub> production in fungi producing AAO. Although veratric acid itself cannot maintain a redox cycle, its reduction by

AADD is the first step for  $H_2O_2$  generation via veratraldehyde redox cycling.

On the basis of the results presented in this paper, the previous scheme of the  $H_2O_2$ -producing system of *P. eryngii* (17) should be slightly modified. In that scheme, it was proposed that two successive reductive reactions of benzoic acid occurred before any oxidative reaction. In other words, the extracellular benzaldehyde found during incubation of *P. eryngii* with benzoic acid was thought to be the result of the successive action of AADD, AAD, and AAO. However, as illustrated in Fig. 2C, the extracellular veratraldehyde derived from veratric acid is directly produced by AADD, since no  $H_2O_2$ , evidencing AAO activity, is shown. Thus, under the incubation conditions used in our studies, the existence of two redox cycles involving the couples acid-aldehyde and aldehyde-alcohol is more likely for those series of compounds whose aldehydes can be oxidized by AAO.

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